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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

One of the most defining clinical features of the Neurofibromatosis type 1 (NF1) disease is the widespread occurrence of multiple benign, peripheral nerve tumors, called neurofibromas, which in some cases can further progress to malignancies. A well-defined explanation of the development of neurofibromas at the molecular level is still lacking. Whereas deregulation of the Ras-mediated signaling pathway contributes to the pathology of NF1, evidence for the involvement of Ras-independent pathways has also been provided. The nature of these pathways remains however to be defined. To gain more insights into the identity of these signaling pathways, we proposed to utilize cDNA microarrays to profile the expression patterns of NF1-associated tumor cells. We set out to isolate neurofibroma and wild type Schwann cells derived from different patients. cDNA representations prepared from these cells will be used as pairs in our profiling experiments using a microarray chip containing 25,000 human cDNA probes. In addition, it is our intent to perform profiling experiments using neurofibroma and wild type Schwann cells inducibly expressing NF1 to assure that the differences observed are indeed due to NF1. The generation of neurofibroma and wild type Schwann cells inducibly expressing NF1 is currently underway.

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ISOLATION AND CHARACTERIZATION OF NF1 TARGET GENES.

INTRODUCTION

Neurofibromatosis type 1 (NF1), also called von Recklinghausen Neurofibromatosis, is a very common autosomal disorder, affecting approximately 1 in 3500 individuals. NF1 is characterized by a diverse spectrum of clinical manifestations, including pigmentation defects called café au lait spots, axillary and inguinal freckling, hamartomas of the iris (Lish nodules), and as one of the most defining clinical features, the development of neurofibromas (2,11,12,16). The latter are benign peripheral nerve sheath tumors. A peculiarity of neurofibromas is their cellular heterogeneity; they consist of a mixture of cells including axonal processes, Schwann cells, fibroblasts, perineurial cells, and mast cells (13,14,18). In some cases, neurofibromas can further progress to malignancies (reviewed in 2).

The gene responsible for Neurofibromatosis type 1 (called NF-1) has been identified and several lines of evidence indicate that NF1 is a tumor suppressor (reviewed in 2). Furthermore, evidence has been provided suggesting that Schwann cells are the initiating cell type in neurofibroma formation (7,13). More recently, elegant studies in mice demonstrated that loss of both alleles of NF1 (*Nf1-/-*) is an obligate step in the development of neurofibromas and that loss of NF1 in the Schwann cell lineage is sufficient to generate tumors (3,17,19).

A major challenge remains to define the signaling pathways NF-1 is acting upon. Knowing the identity of these pathways will provide insights into the molecular basis underlying the development of neurofibromas and will be crucial for the development of novel therapeutic approaches. One pathway NF1 has been shown to act upon is the Ras/Raf/MAPK pathway, a key signal transduction pathway which plays a crucial role in cell growth control (reviewed in 2). In particular, NF1 has been shown to accelerate hydrolysis of Ras-bound GDP to GTP. Hence, loss of NF1 results in elevated levels of activated Ras. However, several lines of evidence indicate that neurofibromin also has functions unrelated to Ras regulation, and support a tumor suppressor role for NF1 independent of its Ras GTPase accelerating activity (1,4,5,8,15). The nature of these pathways remains, however, to be defined. To gain more insights into the identity of these signaling pathways, we proposed to utilize cDNA microarrays to profile the expression patterns of NF1-associated tumor cells. The progress we made over the past year towards achieving this goal is outlined below.

BODY

Given that Schwann cells are the initiating cell type in neurofibroma formation, we opted to make use of Schwann cells isolated from neurofibromas derived from NF1 patients and Schwann cells derived from healthy individuals as paired samples in our profiling experiments. The preparation of isolated Schwann cells is done in collaboration with Dr. R. Foster (Massachusetts General Hospital, Boston) (see a below). In addition, we proposed to perform profiling experiments using neurofibroma and wild type Schwann cells inducibly expressing NF1, to assure that the differences observed are indeed due to NF1. As discussed in b below, we already undertook several steps towards the establishment of inducible NF1 cell lines. Finally, we invested efforts in optimizing conditions for the microarray experiments (see c).

### a) Isolation of normal and neurofibroma derived Schwann cells.

In collaboration with Dr. Foster, we succeeded in isolating Schwann cells form benign dermal and plexiform neurofibromas of 20 different NF1 patients. To this end, the tumors were treated enzymatically to release cells from the tumor matrix. Subsequently, Schwann cells were removed from the heterogeneous mixture through the use of monoclonal antibodies directed against a protein that is only expressed on the surface of Schwann cells, which are coupled to magnetic beads. The isolated Schwann cells were then resuspended in DMEM medium containing 10% fetal calf serum (FCS), 0.5 µM forskolin, 10nM β-heregulin, and 2.5µg/ml insulin (6) and seeded on plates coated with poly-L-lysine. Immunostaining using an antibody against the Schwann cell marker protein S100 of the separated cell population indicated that there was little (less than 5%) contamination in the Schwann cell cultures with other cell types. mRNA was isolated from Schwann cell cultures derived from the 20 different NF1 patients using an 'Ambion mRNA extraction kit' and mRNA extracted from each sample was stored in ethanol at –70°C. As a next step, we will need to isolate wild type Schwann cells from peripheral nerves of at least 4 different organ donors. As discussed in the original proposal, to minimize differences due to genetic background, we want to compare in our profiling experiments representations from NF1 patient cells to a representation of one reference sample. This reference sample will be prepared by pooling representations derived from Schwann cells from at least 4 healthy individuals. The access to peripheral nerves of healthy individuals has, however, been limited.

As soon as a sufficient number of samples derived from NF1 wild type Schwann cells is available, representations will be prepared from the mRNA samples isolated from neurofibroma and wild type Schwann cells and used in the microarray profiling experiments.

b) Setting up an inducible expression system.

To construct inducible NF1 Schwann cell lines, we proposed to make use of the two most widely utilized mammalian inducible systems, namely the tetracycline and ecdysone systems. The tetracycline system is based on an artificial promotor consisting of a tetracycline-response element (TRE) coupled to a CMV minimal promotor, which drives expression of a gene of interest in a tetracycline-dependent manner (20). The ecdysone-inducible mammalian expression system has two components, namely the "sensor", made of two nuclear receptors/transcription factors (VgEcR and RxR) which dimerize in the presence of "inducer" (muristirone A) and activate transcription of the gene of interest under the control of an inducible promotor (9).

As a first step, we have cloned the NF1 cDNA into appropriate retroviral based vectors for each system. We used the PI-TK-Hygro vector for the ecdysone system and the pRev-TRE vector for the tetracycline system (see original application, and Fig 1).

—Pur^R —TRE *PminCMV* · NF1 —poly A—   —U3 ·R·U5·gag· gre HSmin · NF1 —Hyg^R —R·U5

pRev-TRE   **PI-TK-Hygro**
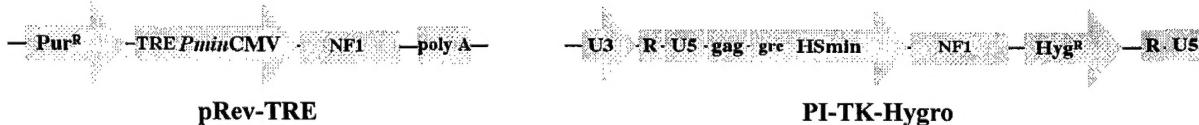
We received the full-length NF1 cDNA in a pBlueskript vector from Dr. A. Bernards

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(Massachusetts General Hospital Cancer Center and Harvard Medical School, Boston). The cloning of full-length NF1 in the above retroviral vectors has been very labor intensive and time consuming, mainly because of the large size of the NF1 transcript and the instability of the recombinant construct in *E. coli*. On advice of Dr. A. Bernards, the amplification of the DNA was performed in the *E. Coli* strain, HB101, to increase the stability of the DNA. The cloning of NF1 was done in three steps, making use of PCR amplifications of some portions of the NF1 gene. The final NF1 constructs have been subjected to sequencing to assure no mistakes were incorporated. These vectors are now ready to be transfected into LinX cells (an amphotropic packaging line) to produce viruses, which will be used to infect the appropriate host Schwann cells, once they are ready. Regarding the generation of suitable host Schwann cells, we set out to introduce the pTet-off regulatory construct that expresses the tetracycline-controlled activator, tTA (tetracycline system) and the VgEcR and RxR receptor constructs (ecdysone system) into wild type and neurofibroma Schwann cells. Viruses carrying the respective receptor genes have been produced. The latter will be used to infect the above mentioned cells, as well as the human neurofibrosarcoma cell line, ST88-14. Once single cell clones are obtained, we will expand these clones and test them for their suitability for appropriate induction making use of a GFP construct.

c) Optimizing conditions for microarray experiments

We proposed to make use in our profiling experiments of chips containing 14,000 human sequence verified cDNA probes. However, more recently, the Cold Spring Harbor Microarray Facility developed chips containing 25,000 cDNA probes. To assure that such size chips will not provide any problems with hybridizations in our hands, we performed several test experiments that allowed us to optimize the hybridization conditions (see below). This optimization is of utmost importance given that obtaining primary Schwann cells from NF1 and healthy individuals is not a trivial undertaking as already discussed in (a), and thus we want to avoid wasting precious material as much as possible.

In our test experiments, we employed cell lines which we were using for other another project in the lab; namely Mo7 and Mo7/p210$^{bcr-abl}$ expressing cells. Specifically, representations prepared from the above cell lines were labeled with Cy3 and Cy5 respectively using Klenow enzyme. The labeled representations were then hybridyzed to the chip containing 25,000 cDNA probes and scanned using an Axon GenePix 4000A scanner. We succeeded in optimizing the hybridization conditions by altering salt concentrations and the hybridization temperature. A scatter plot of one of our optimized profiling experiments is shown in Fig. 1. As can be seen, the majority of the features is centered around $\log_2[R/G]$ value 0 (ratio value equal to 1), and this corresponds to genes whose expression profiles are not altered. The features that possess a $\log_2[R/G]$ value higher than 1 (ratio value higher than 2) depict candidate genes whose expressions are upregulated by p210$^{bcr-abl}$, whereas those with a $\log_2[R/G]$ value below -1 (ratio value lower than 2) represent candidate genes downregulated by p210$^{bcr-abl}$.

KEY RESEARCH ACCOMPLISHMENTS

1. Preparation of Schwann cells derived form benign dermal and plexiform neurofibromas of 20 different NF1 patients and successful isolation of mRNA from these cells.
2. Construction of PI-TK-Hygro-NF1 and pRev-TRE-NF1 constructs, which are essential for

the generation of inducible NF1 Schwann cell lines.

3. Optimization of conditions for microarray experiments which will allow us to reliably use chips containing approximately 25.000 human cDNA probes per slide for hybridizations.

CONCLUSIONS

To gain more insights into the identity of the signaling pathways acting downstream of NF1, we proposed to utilize cDNA microarrays to profile the expression patterns of NF1-associated tumor cells. We decided to make use of normal and neurofibroma derived Schwann cells in our profiling experiments, given that Schwann cells have been shown to be the initiating cell type in neurofibroma formation. We have already isolated mRNA from Schwann cells derived from benign dermal and plexiform neurofibromas of 20 different NF1 patients. Once a sufficient number of wild type Schwann cells are available, representations will be prepared, and representations prepared from neurofibromas will be compared against pooled representations prepared from normal Schwann cells. In addition, we have made progress towards generating NF1 inducible Schwann cells. The latter will allow us to compare samples (induced versus uninduced) that are paired as ideally as possible in the microarray profiling experiments. For the microarray experiments, we worked out the conditions which enables us to reliable use chips containing approximately 25.000 human cDNA probes per slide.

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APPENDICES

One figure

Log$_2$[R/G]

-2 -1 0 1 2
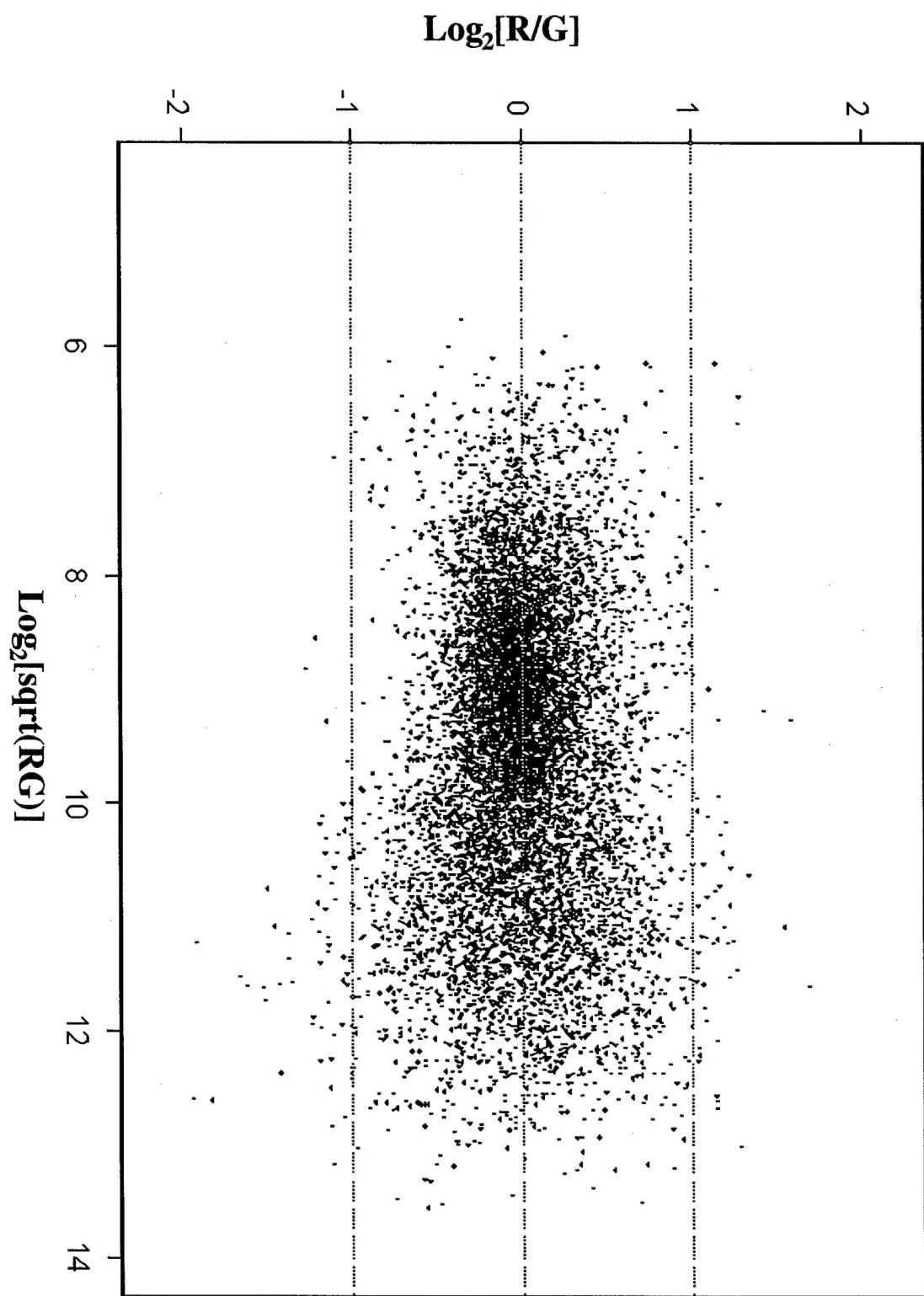
6 8 10 12 14

Log$_2$[sqrt(RG)]

Figure 1: Cy5 labeled representations isolated from Mo7/p210 cells were compared to Cy3 labeled representations isolated from Mo7 cells. The X-axis is the log base two of the geometric mean or square root of the intensity of one channel multiplied by the intensity of the other channel. The Y-axis is the log base two of the ratio of Cy5 to Cy3 (R/G) with background subtraction. Limits are shown to define data points at log2[R/G] values of +1 and -1.